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TITLE: Targeting Neutrophil Protease-Mediated Degradation of Tsp-1 to Induce Metastatic Dormancy

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14. ABSTRACT

Background.
External pre-existing inflammation in the lungs is linked to increased incidence of metastasis. Inflammation –mediated by bacterial infection or cigarette smoke enhanced pulmonary metastasis from breast cancer in humans and mice. Similarly, autoimmune arthritis, characterized by increased recruitment of inflammatory neutrophils and macrophages in the lungs was associated with increased breast cancer metastasis to the lungs. Despite this compelling link between inflammation and metastasis, the mechanisms by which inflammation contributes to tumor outgrowth in distant metastatic organs have remained underexplored. We believe that targeting inflammation-mediated metastasis has tremendous potential in the treatment of high-risk breast cancer patients.
Overarching challenges. Breast cancer affects more than 1.7 million individuals a year worldwide, with approximately 500,000 deaths. Importantly, >90% of this mortality is a consequence of metastatic disease that is resistant to adjuvant therapies. Despite this clinical significance, there is a conspicuous lack of a single FDA approved molecularly targeted anti-metastatic therapy. Hence, there is an urgent medical need to develop new targeted anti-metastatic therapeutic approaches. However, a lack of mechanistic understanding by which tumor cell colonize and outgrow in distant metastatic organs, has been a major impediment to the development of an effective anti-metastatic therapy.
Hypothesis /Objective. We hypothesize that intervention against inflammation-driven neutrophil elastase (NE)/Cathepsin G (CG)-Thrombospondin-1 (Tsp-1) axis can be developed into an anti-metastatic therapy in breast cancer. Our objectives are: 1) to establish that the neutrophil NE/CG-Tsp-1 axis is the dominant pathway in inflammation-mediated metastasis, 2) to determine the molecular mechanisms by which neutrophil CG/NE-Tsp-1 axis promotes metastasis, 3) to show that NE/CG-Tsp-1 axis modulates Tsp-1-mediated metastatic dormancy, 4) to assess whether pharmacological inhibition of CG/NE can be used to inhibit metastasis, and 5) to determine if induction of Tsp-1 expression in the lung microenvironment with a novel DWLPK peptide constitutes an anti-metastatic approach. Our overall goal is to develop a mechanism-guided intervention against inflammation-driven breast cancer metastasis.
Specific Aims. 1) To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung; 2) To determine if pharmacological inhibition of NE and CG can be used to inhibit metastasis, and 3) To determine if ectopic induction of Tsp-1 expression in the lung microenvironment blocks NE/CG-mediated metastasis.
Study Design. We have recently demonstrated that external inflammation in the lungs is associated with increased incidence of metastasis. We discovered a novel mechanism, whereby abundant neutrophils recruited in the inflamed lungs degranulate their azurophilic granules to release two key serine proteases, CG and NE. These proteases specifically target the tumor suppressor Tsp-1, for proteolysis, to generate tumor-promoting microenvironments. Using a combination of genetic and pharmacological approaches, we will determine the mechanistic role and therapeutic potential of CG/NE-Tsp-1 axis in inflammation-mediated breast cancer metastasis.
Innovation. This proposal addresses the critical and unique link between pre-existing inflammation in the lungs and increased incidence of metastasis from breast cancer. A variety of mouse genetic models, together with compartment-specific gene knockout strategies will be employed. In parallel, pharmacological approaches will be used to complement the genetic strategies, and to provide feasibility for clinical translation. This study emphasizes that therapy should be targeted against the reprogrammed host microenvironment, which contributes to, and supports, the e growth and survival of disseminated tumor cells
Impact. We expect to unravel mechanistic and therapeutic insights and generate unique translational opportunities and may lead to the design of future clinical trials for high-risk breast cancer patients that exhibit inflammation (Cigarette smoke, COPD/emphysema related). Notably, the dual NE/CG protease inhibitor Sivelestat is available and is currently being used in Phase III clinical trials of acute lung injury with systemic inflammatory response syndrome. We expect that findings from our studies will support the potential for repurposing Sivelestat as a dual protease antagonist in the treatment of metastasis in breast cancer patients with lung inflammation. Similarly, induction of Tsp-1 expression with a novel DWLPK peptide drug either alone or in combination with Sivelestat has tremendous potential for designing future clinical trials for high-risk breast cancer patients.

15. SUBJECT TERMS
Triple negative breast cancer, metastasis, lipopolysaccharide, thrombospondin 1, cathepsin G, bone marrow transplantation, neutrophil elastase, sivelestat

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Table of Contents

Page

1. INTRODUCTION:

We hypothesize that intervention against inflammation-driven NE/CG- Tsp-1 axis can be developed into an anti-metastatic therapy in breast cancer. Using a combination of genetic and pharmacological approaches, we propose to achieve the following objectives; 1) to establish that the neutrophil NE/CG-Tsp-1 axis is the dominant pathway in inflammation-mediated metastasis, 2) to determine the molecular mechanisms by which neutrophil CG/NE-Tsp-1 axis promotes metastasis, 3) to show that NE/CG-Tsp-1 axis modulates Tsp-1-mediated metastatic dormancy, 4) to assess whether pharmacological inhibition of CG/NE with Sivelestat can be used to inhibit metastasis, and 5) to determine if induction of Tsp-1 expression in the lung microenvironment with a novel DWLPK peptide constitutes an anti-metastatic approach.

This project addresses BCRP overarching challenges of revolutionizing treatment regimens by replacing interventions that have life-threatening toxicities with ones that are safe and effective; and for advancing the field towards the elimination of mortality associated with metastasis in high-risk breast cancer patients. It also addresses metastatic dormancy, and progression of breast cancer to life threatening metastasis. In summary, we anticipate that the proposed studies will lead to exciting and novel findings that have the potential to impact inflammation-mediated metastasis in breast cancer.

2. KEYWORDS:

breast cancer, metastasis, Thrombospondin 1, neutrophil, inflammation, metastases

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim1: To determine the role of neutrophil NE/CG-Tsp-1 axis in breast cancer metastasis to the lung.

Major Task 1: Determine if the metastasis-suppressive phenotype in NE-/-CG-/- mice can be rescued in Tsp-1-/- mice.

Subtask 1: Generate cohorts of WT, Tsp-1-/- and TKO BMT mice
Subtask 2: Generate LPS-mediated inflammation in WT, Tsp-1-/- and NE-/- CG-/- Tsp-1-/- mice, administer tumor cells (EO771& PyMT).
Subtask 3: Resect primary tumors and evaluate metastasis in lungs. Characterize phenotypes. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X repeat expt): 180 mice

Major Task 2: Determine whether loss of NE/CG-Tsp-1axis impacts metastasis by regulating angiogenesis, or proliferation/apoptosis of tumor cells via Tsp-1 receptor CD36.

Subtask 1: Generate shRNA-mediated loss of CD36 expression in tumor cells
Subtask 2: Administer WT and shRNA- tumor cells into WT, Tsp-1-/- and NE-/- CG-/- BMT mice. Animals: (n=15 per cohort × 3 cohorts × 2 tumor models X 2shRNA X repeat): 360 mice

Major Task 3: Determine if Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-1-/- and NE-/- CG-/- BMT mice. (n=15 per cohort × 3 cohorts × 2 tumor models X repeat): 180 mice

Milestone(s) Achieved: Generation of TKO mice, establish role of NE/CG-Tsp-1 axis. CD36 receptor in inflammation-mediated metastasis, Metastatic dormancy

Aim 2: To determine if pharmacological inhibition of NE and CG can be used to inhibit metastasis.

Major Task 1: Pharmacological inhibition of NE/CG with Sivelestat in WT, Tsp-1-/- NE-/-CG-/- and TKO BMT mice. (n=15 per cohort × 4 cohorts × 2 tumor models X repeat): 240 mice

Major Task 2: Efficacy of Tsp-1-mimetic peptide in inhibiting angiogenesis Tsp-1 deficient lungs. ABT-510 peptide in inhibiting angiogenesis. (n=15 per cohort × 4 cohorts × 2 tumor models X repeat): 240 mice

Milestone(s) Achieved: Determine pharmacological efficacy of Sivelestat and ABT-510 in inflammation-mediated metastasis.

Aim 3: To determine if induction of Tsp-1 expression in the lung microenvironment blocks NE/CG-mediated metastasis.

Major Task 1: Evaluate efficacy of DWLPK peptide in WT, Tsp-1-/- and NE-/- CG-/- BMT mice (n=15 cohort × 3 cohorts × 2 tumor models X repeat): 180 mice+ 30 (scramble controls)= 210 mice
Combine DWLPK with Sivelestat in WT LPS challenged cohorts only (n=15 per cohort × 3 cohorts × 2 tumor models X repeat): 360 mice

Milestone(s) Achieved: Demonstrate efficacy of DWLPK and combined DWLPK and sivelestat in metastasis.

What was accomplished under these goals?

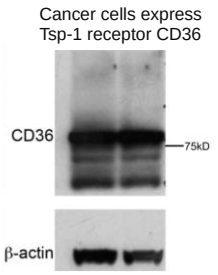
For this reporting period, we are reporting progress for the following:

Subtask 1.1

Given that the generation of triple knockout mice needs a elaborate breeding strategy, we have planned and performed the breeding and have obtained TKO mice (NE-/-CG-/-Tsp1-/-) in preparation of all mouse work once we are ready and ACURO approval has been obtained. (Note: No DoD funds were used for the planning and breeding of these mice as ACURO approval had not been obtained).

Subtask 2.1: Generate shRNA-mediated loss of CD36 expression in tumor cells

We show increased CD36 in tumor cells (Fig. 1A). We have decided to use CRISPR-Cas9 mediated gene knockout as it results in complete depletion of gene products. The sequences for gRNAs (Fig. 1B) have been sent for oligo synthesis. These will be cloned into viral vectors and infected into tumor cells with Cas9 expression vector.



Gene	Oligo ID	Sequence
CD36	MGLibA_09147	CAGAGTTCGTTATCTAGCCA
Cd36	MGLibA_09148	TATCCTATAAGTACCACAGT
Cd36	MGLibA_09149	GCCATAATTGAGTCCTATAA
Cd36	MGLibB_09147	TGTGCAAAACCCAGATGACG
Cd36	MGLibB_09148	CAGTACAATGACACTGTAGA
Cd36	MGLibB_09149	AAATATAACTCAGGACCCCG

Fig. 1. (A) Tsp-1 receptor CD36 is expressed by tumor cells.
(B) Design of CRISPR-Cas9 gRNAs for generating biallelic depletion of CD36

Given that neutrophil degranulation mediates release of proteases neutrophil elastase (NE) and cathepsin G (CG), we have also established a flow cytometry method to determine degranulation of neutrophils. In this assay, we monitor measuring cell surface presentation of the azurophilic granule membrane molecule, CD63. Increased neutrophil degranulation was associated with enhanced presentation of CD63 (Fig. 2)

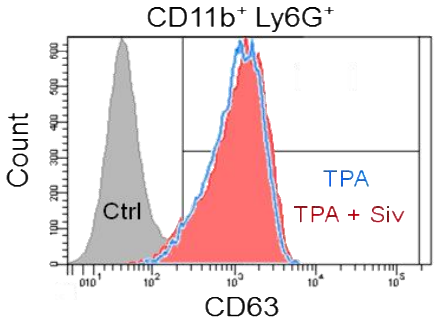


Fig 2. Representative flow cytometry analysis of degranulation marker CD63, in CD11b⁺ Ly6G⁺ cells cultured *in vitro* with 0.01% DMSO (Ctrl, solid grey histogram), 20 nM TPA (TPA, empty blue histogram), or 20 nM TPA + 0.05 µg/µl Sivelestat (TPA + Siv, solid red histogram). Repeated 3 times with similar results.

Gr1+ cells are short-lived *in vitro*, hence their viability in culture might be a limiting factor to the study of Tsp-1 induction. To enhance Gr1+ cell viability, we cultured Gr1+ cells in the presence of cytokines like G-CSF or GM-CSF, which enhance viability of Gr1+ cells *in vitro* for several days. Moreover, we also co-cultured isolated Gr1+ cells on a feeder layer of E4ORF1+ HUVECS, which is a system that allows the expansion of hematopoietic stem cells (HSCs) and CD11b+ Gr1+ cells enhanced viability after 2 days in culture. This system will provide us an opportunity to study metastatic pathways in a highly viable *in vitro* model and for the generation of stable cells for any future *in vivo* work.

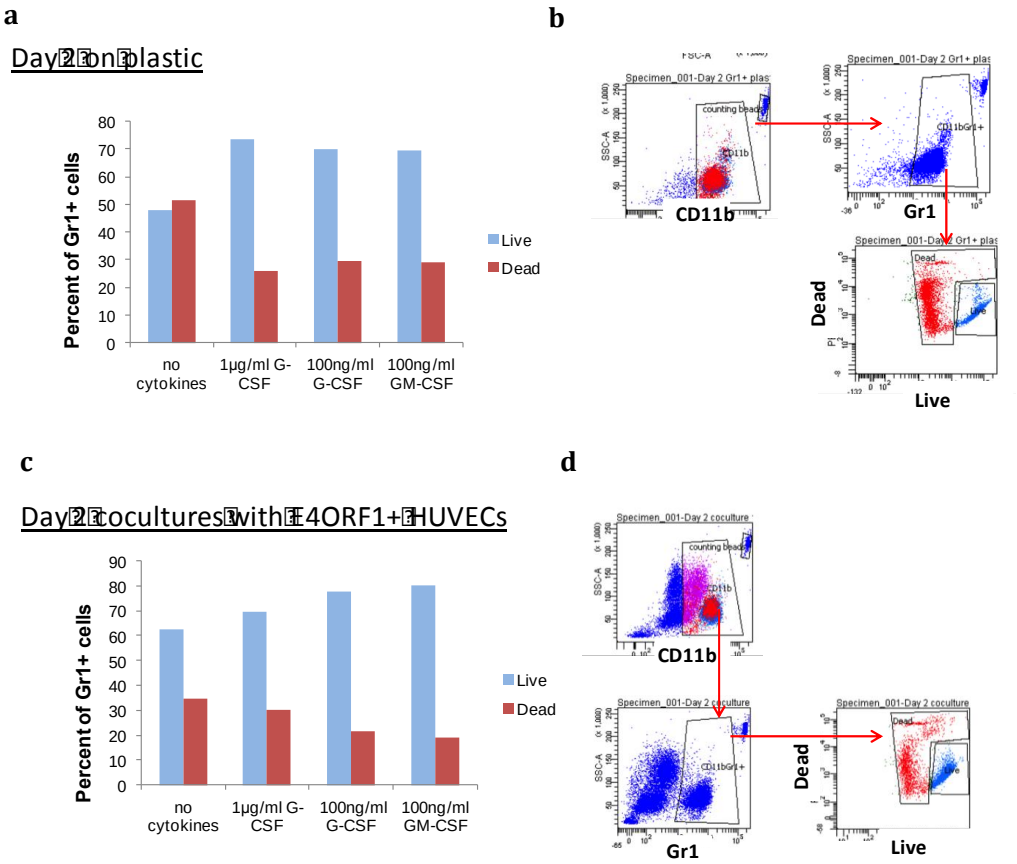


Fig 3: Culturing BM-isolated Gr1+ cells with cytokines on plastic or on a feeder layer of E4ORF1+ HUVECs enhances their viability after 2 days in culture.

a, Percentage of live and dead Gr1+ cells after 2 days in culture on plastic in the absence of added cytokines (no cytokines) or in the presence of 1µg/ml G-CSF, 100ng/ml G-CSF, or 100ng/ml GM-CSF.
b, Representative flow cytometric analysis showing the gates used for CD11b+ Gr1+ cells, and live vs. dead cells to plot the graph in (a).
c, Percentage of live and dead Gr1+ cells after 2 days in coculture with E4ORF1+ HUVECs in the absence of added cytokines (no cytokines) or in the presence of 1µg/ml G-CSF, 100ng/ml G-CSF, or 100ng/ml GM-CSF.
d, Representative flow cytometric analysis showing the gates used for CD11b+ Gr1+ cells, and live vs. dead cells to plot the graph in (c).

Aim 3, Major Task 2: Solubility strategies for the DWLPK peptide were optimized.

What opportunities for training and professional development has the project provided?
Opportunities for training and professional development on the project include the mentorship of post-doctoral associates to help advance their careers.

How were the results disseminated to communities of interest?
In 2016 and 2017, Dr. Mittal has given invited seminars (American Association of Cancer Research Annual Meeting April 2017, University of Nebraska Medical Center Feb 2017, University of Missouri March 2017, Columbus, Metastasis symposium, Cyprus Nov 216) on this topic.

We published the paper that was used as preliminary data for this DOD grant:

Tina El Rayes, Raul Catena, Sharrell Lee, Marcin Stawowczyk, Natasha Joshi, Claudia Fischbach, Charles A. Powell, Andrew J. Dannenberg, Nasser K. Altorki, Dingcheng Gao and **Vivek Mittal**. Lung inflammation promotes metastasis through neutrophil protease-mediated degradation of Tsp-1. **Proc Natl Acad Sci U S A.** 2015 Dec 29;112(52).

This paper described the modified DWLPK pepide that will be used in Aim 3 of the proposal.

Wang S, Blois A, El Rayes T, Liu JF, Hirsch MS, Gravdal K, Palakurthi S, Bielenberg DR, Akslen LA, Drapkin R, **Mittal V**, Watnick RS. Development of a therapeutic cyclic peptide that targets ovarian cancer via the tumor microenvironment (2016) **Sci Transl Med**. 2016 Mar 9; 8(329): 329ra34

What do you plan to do during the next reporting period to accomplish the goals?

As soon as we obtain ACURO Approval, we will initiate in vivo experiments to determine:
1) If if the metastasis-suppressive phenotype in NE-/-CG-/- mice can be rescued in Tsp-1-/- mice.

2) Whether loss of NE/CG-Tsp-1axis impacts metastasis by regulating angiogenesis, or proliferation/apoptosis of tumor cells via Tsp-1 receptor CD36.

3) If Tsp-1 in the lung modulates metastatic dormancy in WT, Tsp-$1^{-/-}$ and $NE^{-/-}$ $CG^{-/-}$ BMT mice.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?
In year 1, we have focused on generating reagents and strategies for the planned in vivo studies. In year 2, we anticipate that the proposed studies will lead to exciting and novel findings that have the potential to finally impact inflammation-mediated metastasis in breast cancer

What was the impact on other disciplines?
Progress in elucidating inflammation-mediated metastasis pathways is likely to attract many investigators across disciplines in breast cancer research and result in rapid advancements towards finding a potential therapy against metastatic breast cancer.

What was the impact on technology transfer?
Nothing to report

What was the impact on society beyond science and technology?
Nothing to report

5. CHANGES/PROBLEMS:

For Aim 3, Task 1.3: As we have experienced problems with the solubility of the DWLPK peptide, better strategies for the DWLPK peptide solubility were created and optimized.

For Aim 1, Subtask 2.1: We have decided to use CRISPR-Cas9 as they are more potent in generating gene knockouts. The sequences for gRNAs are designed and sent for oligo synthesis. We have also developed methods for neutrophil degranuation and for increasing the viability of GR1+ neutrophils in vitro for further experiments.

6. PRODUCTS:

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Vivek Mittal (PD/PI) – 15% Effort*
Project Role:	*PD/PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	*Dr. Mittal led the project and oversaw all aspects of the strategy for planning experiments, etc.*
Funding Support:	

Name:	*Divya Ramchandani, PhD (Post-Doc) – 50% Effort*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Ramchandani has performed all neutrophil degranulation assays and flow cytometry work*
Funding Support:	

Name:	*Robert Bednarczyk (Post-Doc) – 30% effort*
Project Role:	*Post-Doc*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3.6*
Contribution to Project:	*Dr. Bednarczyk has worked on the CRISPR-Cas9 in vitro system.*
Funding Support:	

Name:	*Sharrell Lee (Technician) – 50% effort*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Ms. Lee has assisted Dr. Ramchandani and Dr. Bednarczyk on the in vitro work and has developed the breeding strategies for the triple knockout mice that will be used in future.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No

What other organizations were involved as partners?

None

8. **SPECIAL REPORTING REQUIREMENTS**
   Nothing to report

9. **APPENDICES:**
   Nothing to report